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(54) Title: USE OF POLYMERIZED HEMOGLOBIN TO INCREASE THE ANTITUMOR EFFECT OF THE IONIZING RADIATION

(57) Abstract

A method is disclosed treating a tumor in a host by administering an ultrapurified polymerized hemoglobin solution to the host and thereafter administering ionizing radiation, such as x-ray, to the tumor. In a particularly preferred embodiment, the hemoglobin is bovine hemoglobin.

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Use of polymerized hemoglobin to increase the antitumor effect of the ionizing radiation

Description

Background of the Invention

Solid tumor masses in cancer patients have been found to be heterogeneous in oxygenation and to contain regions of hypoxia. See Vaupel. P., "Oxygenation of Human Tumors", Strahlenther. Onkol. 166:377-386 (1990); and Adams, G.E., The Clinical Relevance of Tumour Hypoxia, 26(4):420-421 (1990). Recent studies in human tumors with oxygen electrodes have reaffirmed the occurrence of significant hypoxic areas within human tumors. Vaupel, P. ibid; Kallinowski, F. et al., "Tumor Tissue Oxygenation as Evaluation by Computerized-pO_2-Histogragphy", Int. J. Radiat. Oncol. Biol. Phys. 19:953-961 (1990); and Gatenby, R.A. et al., "Oxygen Distribution in Squamous Cell Carcinoma Metastases and Its Relationship to Outcome of Radiation Therapy", Int. J. Radiat. Oncol. Biol. Phys. 14:831-838 (1988). Preclinical studies, both in vitro and in vivo, have established that hypoxia protects tumor cells from the cytoxic actions of radiation and chemotherapeutic agents and thereby may be a significant factor in therapeutic resistance. Adams, G.E. ibid; Sartorelli, A.C., "Therapeutic Attack of Hypoxic Cells of Solid Tumors: Presidential Address", Cancer Res. 48:775-778 (1988); Teicher, B.A. et al., "Classification of Antineoplastic Agents by Their Selective Toxicities Toward Oxygenated and Hypoxic

Tumor Cells", Cancer Res. 41:73-81 (1981); and Teicher, B.A. et al., "Classification of Antineoplastic Treatment by Their Differential Toxicity Toward Putative Oxygenated and Hypoxic Tumor Subpopulations in vivo in the FSaIIC Murine Fibrosarcoma", Cancer Res. 503339-3344 (1990).

Increased delivery of oxygen from the lungs can be a useful way of improving the oxygenation of solid tumor masses by altering the gradient of oxygen as it is absorbed from the vasculature and distributed into the tissue. Because of this, one strategy which has been attempted to overcome the problem of hypoxia in treating tumors involves the use of perfluorocarbon emulsions with oxygen or carbogen (95% oxygen/5% carbon dioxide) breathing. Holden, S.A. et al., "Addition of a Hypoxic Cell Selective Cytotoxic Agent (mitomycin C or porfiromycin) to Treatment with Fluosol-DA® /Carbogen/Radiation", Radiother. Oncol. 18:59-70 (1990); Teicher, B.A. et al., "The Effect of Fluosol-DA and Oxygenation Status on the Activity of Cyclophosphamide In Vivo" Cancer Chemother. Pharmacol. 21:286-291 (1988); Martin, D.F. et al., "Enhancement of Tumor Radiation Response by the Combination of a Perfluorochemical Emulsion and Hyperbaric Oxygen", Int. J. Radiat. Oncol. Biol. Phys. 13: 747-751 (1987); Teicher, B.A. and C.M. Rose, Perfluorochemical Emulsion Can Increase Tumor Radiosensitivity" Science 223:934-936 (1984); and Teicher, B.A. et al., "Optimization of Perfluorochemical Levels with Radiation Therapy"

Cancer Res. 49:2693-2697 (1989). In preclinical solid tumor models, the use of perfluorocarbon emulsions with carbogen or oxygen breathing in conjuction with radiation therapy has produced positive results. Teicher, B.A. and C. M. Rose, ibid; Teicher, B.A. et al., ibid; Teicher, B.A. and C. M. Rose, "Oxygen-Carrying Perfluorochemical Emulsion as an Adjuvant to Radiation Therapy in Mice", Cancer Res. 44:4285-4288 (1984); Teicher, B.A. and C.M. Rose, "Effect of Dose and Scheduling on Growth Delay of the Lewis Lung Carcinoma Produced by the Perfluorochemical Emulsion, Fluosol-DA", Int. J. Radiat. Oncol. Biol. Phys. 12:1311-1313 (1986); Teicher, B.A., et al., "Influence of Scheduling Dose and Volume of Administration of the Perfluorochemical Emulsion Therox® on Tumor Response to Radiation Therapy", Int. J. Radiat. Oncol. Biol. Phys., in press (1991); Teicher, B.A. et al., "Effect of Fluosol®-DA on the Response of Intracranial 9L Tumors to X-rays and BCNU", Int. J. Radiat. Oncol. Biol. Phys. 15:1187-1192 (1988); Lee, I. et al., "Effects of Fluosol-DA and Carbogen on the Radioresponse of SCK Tumors and Skin of A/J Mice". Radiat. Res. 112:173-182 (1987); Martin, D.F. et al., "Effect of a Perfluorochemical Emulsion on the Radiation Response of BA 1112 Rhabdomyosarcomas", Radiat. Res. 112:45-53 (1987); Moulder, J.E. et al., "Applicability of Animal Tumor Data to Cancer Therapy in Humans". Int. J. Radiat. Oncol. Biol. Phys. 14:913-927 (1988); Moulder, J.E. and B.L. Fish,

"Tumor Sensitization by the Intermittent use of Perfluorochemical Emulsions and Carbogen Breathing in Fractionated Radiotherapy", In: E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott (eds.), Proceedings of the 8th International Congress of Radiation Research, Vol. 1, p. 299, London: Taylor and Francis, Inc. (1987); Rockwell, S. et al., "Reactions of Tumors and Normal Tissues in Mice to Irradiation in the Presence and Absence of a Perfluorochemical Emulsion" Int. Radiat. Oncol. Biol. Phys. 112:1315-1318 (1986); Song, C.W. et al., "Increase in pO_2 and Radiosensitivity of Tumors by Fluosol®-DA (20%) and Carbogen", Cancer Res. 47:442-446 (1987); and Zhang, W.L. et al., "Enhancement of Tumor Response to Radiation by Fluosol-DA", Int. J. Radiat. Oncol. Biol. Phys. 10:172-175 (1984).

Further, some initial clinical trials of the perfluorochemical emulsion, Fluosol®-DA and oxygen breathing with radiation therapy have been carried out and some are still underway. Rose, C.M. et al., "A Clinical Trial of Fluosol®-DA 20% in Advanced Squamous Cell Carcinoma of the Head and Neck", Int. J. Radiat. Oncol. Biol. Phys. 12:1325-1327 (1986); Lustig, R. et al., "Phase I-II Study of Fluosol-DA and 100% Oxygen Breathing as an Adjuvant to Radiation in the Treatment of Advanced Squamous Cell Tumors of the Head and Neck", Int. J. Radiat. Oncol. Biol. Phys. 16:1587-1594 (1989); Lustig, R. et al., "Phase I/II Study of Fluosol and 100% Oxygen Breathing as an Adjuvant to Radiation in the Treatment of

Unresectable Non-Small Cell Carcinoma of the Lung", Int. J. Radiat. Oncol. Biol. Phys. 17sl:202 (1989); and Evans, R.G. et al., "A Phase I-II Study of the Use of Fluosol®-DA 20% as an Adjuvant of Radiation Therapy in the Treatment of Primary High-Grade Brain Tumors", Int. J. Radiat. Oncol. Biol. Phys. 1721:175 (1989).

The effect of perfluorocarbon emulsions in carbogen or oxygen breathing with certain chemotherapeutic agents has also been studied in preclinical solid tumor models. Teicher, B.A. et al., "Classification of Antineoplastic Treatments by Their Differential Toxicity Toward Putative Oxygenated and Hypoxic Tumor Subpopulations in vivo in the FSaIIC Murine Fibrosarcoma", Cancer Res. 50:3339-3344 (1990); Holden, S.A. et al., "Addition of a Hypoxic Cell Selective Cytotoxic Agent (Mitomycin C or Porfiromycin) to Treatment with Fluosol-DA®/Carbogen/Radiation", Radiother. Oncol. 18:59-70 (1990); Teicher, B.A. et al., "The Effect of Fluosol-DA and Oxygenation Status on the Activity of Cyclophosphamide in vivo" Cancer Chemother. Pharmocol. 21:286-291 (1988); Teicher, B.A. et al., "Approaches to Defining the Mechanism of Fluosol-DA 20%/Carbogen Enhancement of Melphalan Antitumor Activity", Cancer Res. 47:513-518 (1987); Teicher, B.A. et al., "Differential Enhancement of Melphalan Cytotoxicity in Tumor and Normal t+Tissue by Fluosol-DA and Oxygen Breathing", Int. J. Cancer 36:585-589 (1985); Teicher, B.A. et al., "Effects of Various

Oxygenation Conditions on the Enhancement by Fluosol-DA of Melphalan Antitumor Activity", Cancer Res. 47:5036-5041 (1987); Teicher, B.A. and S.A. Holden, "A Survey of the Effect of Adding Fluosol-DA 20%/O_2 to Treatment with Various Chemotherapeutic Agents", Cancer Treat. Rep. 71:173-177 (1987); Teicher, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol-DA on Cancer Chemotherapeutic Agents", Biomat., Art. Cells and Art. Organs 16:533-546 (1988); Teicher, B.A. et al., "Effect of Oxygen on the Cytotoxicity of Antitumor Activity of Etoposide", J. Natl. Cancer Inst. 75:1129-1133 (1985); Teicher, B.A. et al., "Effect of Fluosol-DA/O_2 on Tumor Cell and Bone Marrow Cytotoxicity of Nitrosoureas in Mice Bearing FSaII Fibrosarcoma", Int. J. Cancer 38:285-288 (1986); Teicher, B.A. et al., "Effect of Fluosol-DA/O_2 on the Antitumor Activity and Pulmonary Toxicity of Bleomycin", Cancer Chemother. Pharmacol. 18:213-218 (1986); Teicher, B.A. et al., "Effects of Fluosol®-DA and Oxygen Breathing on Adriamycin Antitumor Activity and Cardiac Toxicity in Mice", Cancer 61:2196-2201 (1988); Teicher, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol®-DA on the Cytotoxicity and Antitumor Activity of Bleomycin", J. Natl. Cancer Inst. 80:599-603 (1988); Teicher, B.A. et al., "Effect of Fluosol-DA/Carbogen on Etoposide/Alkylating Agents Antitumor Activity", Cancer Chemother. Pharmacol. 21 281-285 (1988); Martin, D.F. et al., "Potentiation of Rat Brain Tumor Therapy by Fluosol and Carbogen", NCI

Monogr. 6:119-122 (1988); and Kim, G.E. and C.W. Song, "The Influence of Fluosol-DA and Carbogen Breathing on the Antitumor Effects of Cyclophosphamide In Vivo", Cancer Chemother. Pharmacol. 25:99-102 (1989). With many chemotherapeutic agents, very positive therapeutic results have been obtained and several initial clinical trials have been carried out with Fluosol-DA and oxygen breathing with single anticancer drugs. See Gruber, M. et al., "Phase I/II Study of Fluosol®/0_2 in Combination with BCNU in Malignant Glioma", Proc. Amer. Assoc. Cancer Res. 31:190 (March 1990); Carewal, H. et al., "Fluosol®/Oxygen in Combination with Cyclophosphamide in Advanced Non-Small Cell Lung Carcinoma (NSCLC): Phase I Results", Proc. Amer. Assoc. Cancer Res. 30:271 (March 1989); and Meyers, F. et al., "Phase I/II Study of Fluosol®/Oxygen in Combination with Weekly 5-Fluorouracil (5FU) in Metastatic Colorectal Carcinoma", Proc. Amer. Assoc. Cancer Res. 30:256 (March 1989).

Despite the initial success with the use of perfluorocarbon emulsions and carbogen or oxygen breathing in conjuction with ionizing radiation, these techniques have not proven entirely satisfactory. For example, perfluorocarbons have very limited oxygen-transport capability at ambient oxygen pressures. Blood delivers approximately 6% (v/v) oxygen to tissues at ambient pressures, whereas, at these same pressures, perfluorocarbon

emulsions can only delivery about 2% (v/v).

Summary of the Invention

This invention relates to a method for treating a tumor in a host, including a human being, with ionizing radiation. In this method, an ultrapurified polymerized hemoglobin solution (UPHS) is administered to the host in an amount which significantly increases the antitumor effect of the radiation. An effective amount of ioninzing radiation is also administered to the host. In particularly perferred embodiment, the hemoglobin is bovine hemoglobin.

Adminstering an ultrapurified polymerized hemoglobin solution with administration of the ionizing radiation significantly increases the antitumor effect of the radiation. In addition, the use of the hemoglobin solution, in contrast to the use of perfluorocarbon emulsions, has certain advantages. Hemoglobin is able to chelate and deliver oxygen under air-breathing conditions. Polymerized hemoglobins have a longer circulating half-life than many of the perfluorocarbon emulsions and, therefore, have a longer functional period post-administration. The acidic environments in tumors increase the off-loading of oxygen and, therefore, the oxygen delivery from hemoglobin, as should temperature elevation (i.e., clinical hypothermia). Hemoglobin solutions also have less retention in

normal tissues, which is a concern with many perfluorocarbon preparations.

Brief Description of the Figures

Figure 1 is a plot of the tumor growth delay of the Lewis lung carcinoma produced by single dose or fractionated radiation treatment with or without previous administration of ultrapurified polymerized bovine hemoglobin solution (UPBHS) and air breathing or carbogen breathing versus radiation dose.

Figure 2 is a plot of the surviving fraction of FSaIIC cells from FSaIIC tumors treated in vivo with single doses of radiation with and without prior administration of UPBHS versus radiation dose.

Figure 3 is a graph of pO_2 measurements made of 13672TB mammary carcinoma, using a histograph.

Figure 4 graphically illustrates that the ultrapurified polymerized bovine hemoglobin solution called Hemopure alters the oxygenation profile of the tumor.

Figure 5 is the pO_2 measurements made of 9L brain tumor using a histograph.

Figure 6 graphically illustrates that the ultrapurified polymerized bovine hemoglobin solution called Hemopure improvwews the oxygenation of the 9L tumor whether the animals were breathing air or carbogen.

Detailed Description of the Invention

This invention relates to a method for treating a tumor in a host. The host can be any species which develops solid tumors. Examples of hosts include, but are not limited to, reptiles, amphibians, avians and mammals, including human beings, as well as domestic animals such as dogs, cats, cows and horses.

Tumors treatable by this method include those in which oxygen heterogeneity, including regions of hypoxia, protect tumor cells against the cytotoxic action of ionizing radiation. These are usually solid tumors, such as sarcomas, carcinomas, lymphomas, etc. However, in certain cases of dispersed tumor cells, such as advances leukemia, masses of tumor cells form which can produce regions of oxygen heterogeneity, as well.

Any type of ionizing radiation which exhibits an antitumor effect can be employed with this invention. Some examples include X-rays, gamma rays, high-energy electrons and High LET radiation, such as protons, neutrons and alpha particles.

The ionizing radiation is employed by techniques well-known to those skilled in the art. For example, X-rays and gamma rays are applied by external and/or interstitial means from linear accelerators or radioactive sources. High energy electrons can be produced by linear accelerators. High LET radiation is also produced by linear accelerators and can also be applied from radioactive sources implanted

interstitially.

Dosages of the ionizing radiation are those conventionally applied in radiotherapeutic treatment of tumors. In certain cases, it might be that use of UPHS in conjunction with ionizing radiation might lower the dosage of ionizing radiation required.

In order to increase oxygen transport to the site of a tumor, an ultrapurified polymerized hemoglobin solution (UPBHS) is administered to the host. Although not essential, it is preferred to administer the UPHS prior to administration of the antitumor agent. Also, the hemoglobin solution is preferably administered intravenously so that it is taken up into the bloodstream of the host immediately.

As mentioned above, it is preferably to administer UPHS prior to administration of the chemotherapeutic agent. The amount of time between the administration of the hemoglobin solution and chemotherapeutic agent will depend upon factors such as the amount of time it takes the hemoglobin solution to be fully incorporated into the circulatory system of the host, the lifetime of the hemoglobin solution, etc. Since polymerized bovine hemoglobin has been found to remain in the host's blood stream for up to at lest 48 hours, anytime during this period is sufficient.

Hemoglobin sufficient for the hemoglobin solutions can be derived from a wide variety of sources. These sources include human blood, such as

outdated blood bank supplies. Additionally, the hemoglobin can be derived from a variety of mammalian sources such as horses, pigs, cows, sheep, etc.

In a preferred embodiment, the hemoglobin will be derived from a species in which the hemoglobin is chloride ion-dependent for oxygen transport rather than dependent upon 2,3-diphosphoglycerate (2,3-DPG) or other phosphate molecules. This is because 2,3-DPG, present in human red blood cells, is not available freely in the circulatory system of the host to effect oxygen uptake and release for hemoglobin solutions administered according to this invention. Thus, it is preferred to employ a hemoglobin which is chloride ion-dependent for oxygen transport, such as those hemoglobins derived from sheep, goats, cows and cats. See Bunn, H.F., "Differences in the Interaction of 2,3-Diphosphoglycerate with Certain Mammalian Hemoglobins", Science 172:1049-50 (1971); Breepoel, P.M. et al., "Interaction of Organic Phosphates with Bovine Hemoglobin -- I Oxylabile and Phosphate Labile Proton Binding", Pflugers Arch. 389:219-225 (1981); and Fronticelli, C. et al., "Solvent Regulation of Oxygen Affinity and Hemoglobin -- Sensitivity of Bovine Hemo-Globin to Chloride Ions", J. Biol. Chem. 259:10841-4 (1984). Bovine hemoglobin is particularly preferred because of its proven ability to transport oxygen in human beings and other mammals, in a chloride ion-dependent way, and because of its low antigenicity in human beings when it has

been ultrapurified.

In order to increase the useful life of hemoglobin in the circulation, it is polymerized or crosslinked by a variety of techniques. Crosslinking agents include dialdehydes, such as glyoxal, malonic dialdehyde, succinic dialdehyde, glutaraldehyde, adipaldehyde, 3-methylglutaraldehyde, propyladipaldehyde, phthalic dialdehyde, terephthaldehyde and malonic dialdehyde have been employed. See, in this regard, Bonsen et al., U.S. Patent Nos. 4,001,200; 4,001,401; and 4,053,590; Bonhard et al., U.S. 4,136,093 and U.S. Patent Nos. 4,336,248; the teachings of each of which are incorporated herein by reference.

The polymerized hemoglobin solution is ultrapurified by various filtration and chromatographic procedures which have been described heretofore in the art. An ultrapure hemoglobin solution, according to this invention, is a hemoglobin solution which is substantially free of stroma, endotoxin, other pyrogenic substances, phospholipids, immunoglobins and cellular-contained enzymes.

A particularly preferred ultrapure polymerized hemoglobin solution is based upon bovine hemoglobin. Such a bovine blood substitute has an endotoxin concentration of less than 0.5 endotoxin units/ml as measured by the LAL test; a phospholipid concentration of less than about 1 nanogram/milliliter and has a molecular weight

distribution greater than 90% in the range of 68,000-500,000 daltons. This bovine hemoglobin solution also has an osmolarity measured by freezing point depression in the range of 180-320 milliosmols per liter; a hemoglobin content of 5-25 grams per deciliter; a met hemoglobin content of less than 20%; a p_{50} in the range of 18-36 mmHg; an intravascular half life of at least two days; a crosslinking profile on gel permeation chromatography of 50-70%.

Such ultrapurified polymerized bovine hemoglobin solution is made and sold by Biopure Corporation, Boston, MA under the trademark Hemopure. This and other ultrapurified hemoglobin solutions are described in International Patent Application PCT/US87/02967, published under WO88/03408, the teachings of which are hereby incorporated by reference.

Appropriate dosages of UPBHS can be determined by those skilled in the art using routine experimentation. The dose employed in the murine studies in the Examples herein was 12 ml/kg, which is 13%-15% of the estimated circulatory volume, or 1.32 g protein/kg. This dose corresponds to 840 ml as the comparative human dose or 17%-19% of estimated circulatory volume, and 92.4 g protein in a 70 kg person. Multiple doses of UPHS, for example one before each radiation treatment, are, of course, useful with this invention and can be preferred in many cases.

Although not required, it is preferred to have

the host breath oxygen-enriched gas prior to and post administration of the ionizing radiation. This can be done by having the host breathe oxygen-enriched air, 100% oxygen or carbogen (95% oxygen/5% CO2), or in certain cases exposing the host to hyperbaric oxygen conditions.

The techniques for treating tumors described herein can be employed at normal body temperatures (hypothermia).

This invention will now be further and more specifically described by the following examples.

Example I

Tumor Growth Delay with UPBHS or Perfluorocarbons Used in Conjuction with Gamma Ray Irradiation

The Lewis lung tumor model was employed. Shipley, W.V. et al., "Tumor Size Dependence in the Radiation Response of the Lewis lung carcinoma", Cancer Res. 35:2488-2493 (1975); Stanley, J.A. et al., "Influence of Tumor Size on Hypoxic Fraction and Therapeutic Sensitivity of Lewis lung Tumor", Br. J. Cancer 36:105-113 (1977); and Steel, G.G. et al., "Combined Radiotherapy-Chemotherapy of Lewis Lung Carcinoma", Int. J. Radiat. oncol. Biol. Phys. 4:49-52 (1978). When the tumors were approximately 100 mm_3 in volume, Fluosol-DA (2.4 g PFC/kg, 0.3 ml), F44E at doses of 2 g PFC/kg (0.2 ml), 4 g PFC/kg (0.2 ml), or 8 g PFC/kg (o.2 ml), or UPBHS (Hemopure blood-substitute solution) (0.3 ml) was injected into

the tail vein of the mice and air or carbogen breathing was begun.

Fluosol-DA 20% was obtained from Alpha Therapeutics Corp., Los Angeles, CA. The stem emulsion consists of 25% (w/v) as a cryoprotective agent. The annex solution (electrolyte/bicarbonate solution) furnished the preparation with physiological osmolarity. The half-life of Fluosol-DA in vivo is about 12 hours. Geyer, R.P., "Substitutes for Blood and Its Components" In: Jamieson, G.A. and T.J. Greenwalt, eds., Blood Substitutes and Plasma Expanders, New York, NY: Liss pp. 1-21 (1978).

The F44E perfluorochemical emulsion, Therox (E.I. DuPont de Nemours & Com., Chemicals and Pigments Dept., Deepwater, NJ), contains 48% (v/v, 83% w/v) F44E and egg yolk lecithin as the emulsifier in an isotonic buffer was used as the perfluorochemical source. The particle size of the emulsion is 0.25 μM. The half-life of this emulsion in circulation is about 2.5 hours and the dwell time of this perfluorochemical in tissues is about 7 days. Riess, J.G., "Reassessment of Criteria for the Selection of Perfluorochemicals for Second-Generation Blood Substitutes", Analysis of Structure/Property Relationships", Artif. Organs (Cleve.) 8:44-56 (1984); and Riess, J.G. and M. LeBlanc, "Solubility and Transport Phenomena in Perfluorochemicals Relevant to Blood Substitution and Other Biomedical Applications", Pure Appl. Chem. 54:2383-2406 (1982).

Hemopure blood-substitute solution is the polymerized form of a highly purified bovine hemoglobin solution. Hemopure solution contains 11± 2 gm/deciliter of bovine hemoglobin. Greater than 50% (w/v) of the hemoglobin has a molecular weight greater than 68,000 and up to 500,000, and less than 10% (w/v) has a molecular weight less than or equal to 68,000. Hemopure solution also contains sodium (120 ± 20 mM/L), chlorine (115 ± 25 mM/l), and potassium (4.0 ± 1 mM/L) in this buffer (pH 7.8 ± 0.4). The circulating half-life of the Hemopure solution is about 2.5 days. DeVenuto, F., "Evaluation of Human and Bovine Modified-Hemoglobin Solution as Oxygen Carrying Fluid for Blood Volume Replacement", Biomaterials, Artificial Cells, and Artificial Organs V. 16, Nos. 1-3:77-84 (1988); and Winslow, R.M., "Optimal Hematologic Variables for Oxygen transport Including P50, Hemoglobin Cooperativity, Hematocrit, Acid-Base Status, and Cardiac Function", Biomaterials, Artificial Cells, and Artificial Organs, V. 16, Nos. 1-3:149-172 (1988).

Carbogen breathing was maintained for 1 hours before and during delivery of each radiation fraction for those groups receiving carbogen, using ^{137}Cs gamma rays locally (Gamma Cell 40, Atomic Energy of Canada, Ltd.) to the tumor-bearing limb (dose rate, 0.88 Gy/min.). The animals were irradiated unanesthetized in an apparatus which allowed local treatment of the tumor-bearing limb. The animals received less than

2% of the total dose whole body. In the fractionated radiation regimen, 2, 3, or 4 Gray were delivery daily for 5 days, ± daily treatment with the various oxygen-carrying solutions. In the single dose radiation regimen, the radiation doses were 10, 20 or 30 Gray. Tumor size was ascertained by thrice-weekly measurements with calipers. The experimental end point was the number of days post-tumor cell implantation for the tumors to reach a volume of 500 mm^3. Schabel, Jr., F.M. et al., "Testing Therapeutic Hypotheses in Mice and Man: Observations on the Therapeutic Activity Against Advanced Solid Tumors of Man", Methods Cancer Res. 17:3-51 (1979). Untreated tumors reach 500 mm^3 in approximately 14 days. Ech experimental group had 7 mice and each experiment was repeated at least once; therefore, the minimum number of tumors examined at each point was 14. Teicher, B.A. and C.M. Rose, "Perfluorochemical Emulsions Can Increase Tumor Radiosensitivity", Science 223:934-936 (1984).

Data from the tumor growth delay experiments were analyzed using a computer program written in BASIC. The program first derives the best-fit curve for each individual set of tumor volume data and then calculates the median, mean, and standard error on the day the tumor reach 500 mm^3. Dose modifying factor was calculated as the ratio of the slopes of the tumor growth delay or tumor cell survival curves in the presence or absence of the perfluorochemical emulsion or PBHS.

The dose modifying factors calculated for these radiation treatment regimens are shown in Table 1.

Dose Modifying Factors Observed in the Lewis Lung Carcinoma Treated with Perfluorochemical Emulsions or with UPBHS and Various Levels of Oxygen[a]

Oxygen Carrier	Dose g/kg	Dose (ml/kg)	% Oxygen Atmosphere 20%	65%	85%	95%
Fluosol-DA	2.4	(12)	1.0[b]	1.0	1.3	2.1
F44E	2.0	(8)	1.0	1.0	1.0	1.9
	4.0	(8)	1.0	1.0	1.0	2.2
	8.0	(8)	1.0	1.25	1.3	1.8
PBHS	1.32	(12)	1.6	--	--	2.1

[a] Dose modifying factors were calculated as the ratio of the slopes of the tumor growth delay curves in the presence or absence of the perfluorochemical emulsions or hemoglobin preparation for animals breathing each atmosphere. X-ray dose were 2, 3, or 4 gy daily for 5 days locally to the tumor. [b]1.0 = no effect.

Fluosol-DA was administered at the optimal dose for that perfluorochemical emulsion of 2.4 g PFC/kg (0.3 ml. 12 ml/kg). Fluosol-DA administration did not significantly alter the response of the tumors to the radiation treatments when air or 65% oxygen was breathed, but there was a small benefit from Fluosol-

DA if an atmosphere of 85% oxygen was breathed. A dose modifying factor of 2.1 was obtained when carbogen (95% O_2, 5% CO^2) was used, indicating substantial enhancement of tumor growth delay with Fluosol-DA/carbogen/radiation compared with carbogen/radiation.

The perfluorochemical emulsion preparation, F44E, is much more concentrated than Fluosol-DA, allowing a dosage range of perfluorochemical to be examined. An F44E dose of 0.2 ml allowed a dose of 8 g PFC/kg to be administered, and dilutions with phosphate buffered 0.9% saline were used to achieve the doses of 5\4 g PFC/kg and 2 g PFC/kg each at 8 ml/kg (i.e., in 0.2 ml). Administration of the two lower doses of the F44E perfluorochemical emulsion did not alter the response of the tumor to the fractioned radiation regimen if air, 65% oxygen, or 85% oxygen was breathed; however, if 95% oxygen was breathed, a dose modifying factor of 1.9 was obtained with 2 g PFC/kg of F44E, and a dose modifying factor of 2.2 was obtained with 4 g PFC/kg of F44E. At the highest dose of perfluorochemical emulsion of 8 g PFC/kg of F44E, although there was no increase in tumor growth delay produced by radiation when air was breathed, and a dose modifying factor of 1.8 was obtained when carbogen was breathed (Table 1).

Daily administration of 1.32 g protein/kg (0.3 ml, 12 ml/kg) of UPBHS along with air breathing and fractionated radiation therapy produced a significant enhancement of tumor growth delay, resulting in a

dose modifying factor of 1.6. When the UPBHS administration was accompanied by carbogen breathing, there was a further increase in tumor growth delay, so that the dose modifying factor of 1.6. When the UPBHS administration was accompanied by carbogen breathing, there was a further increase in tumor growth delay, so that the dose modifying factor increased to 2.2

Shown in Figure 1 is the tumor growth delays of the Lewis lung carcinoma produced by single dose and fractionated radiation and UPBHS under various conditions. When UPBHS either 0.5 ml or 0.3 ml was administered 1 hours before radiation treatment and the animals were maintained with air breathing, a dose modifying factor of 1.5-1.6 was obtained. When the same UPBHS treatments were followed by 1 hours of carbogen breathing before and during radiation delivery, a dose modifying factor of 2.0-2.1 was obtained with 0.5 ml of UPBHS, and a dose modifying factor of 3.8-40 was obtained with 0.3 ml of UPBHS. In effect UPBHS and carbogen breathing resulted in 3.9 ± 0.5 days of tumor growth delay in the absence of radiation treatment. When UPBHS (0.3 ml) was administered on alternate days of a daily fractioned radiation regimen with air breathing, a dose modifying factor of 1.3-1.4 was obtained. However, if UPBHS (0.3 ml) was administered daily 1 hours before each radiation fraction with air breathing, a dose modifying factor of 1.6-1.7 was obtained. If daily carbogen breathing was added to the treatment

with daily UPBHS and fractionated radiation, a dose modifying factor of 2.0-2.2 was obtained.

Example II

Tumor Cell Survival with UPBHS Used in Conjuction with X-Rays

Tumor cell survival assay was carried out using the FsaIIC fibrosarcoma. The FSaII fibrosarcoma (Rice, L. et al., "The Radiosensitivity of a Murine Fibrosarcoma as Measured by Three Cell Survival Assays", Br. J. Cancer 41:240-245 (1980)) adapted for growth in culture (FSaIIC) (Teicher, B.A. and C.M. Rose, "Perfluorochemical Emulsions Can Increase Tumor Radiosensitivity", Science 223:934-936 (1984)) was carried in C3H/FeJ male mice. 2 x 10^6 tumor cells prepared from a breed of several stock tumors were implanted intramuscularly into the legs of C3H/FeJ male mice 8-10 weeks of age.

When the FSaIIC tumors were approximately 100 mm^3 in volume (about 1 week after tumor cell implantation), animals were treated with single doses of X-rays at 5, 10, 15 or 20 Gy alone or preceded by UPBHS (0.3 or 0.5 ml) administered via tail vein injection. Mice were killed 24 hours after treatment to allow for full expression of radiation cytotoxicity and repair of potentially lethal damage and then immersed in 95% ethanol. The tumors were excised under sterile conditions, and single cell suspensions were prepared for the colony-forming

assay. Teicher, B.A. eta l., "Approaches to Defining the Mechanism of Fluosol-DA 20% with Carbogen Enhancement of Melphalan Antitumor Activity", Cancer Res. 47:513-518 (1987); and Teicher, B.A. and C.M. Rose, "Perfluorochemical Emulsions Can Increase Tumor Radiosensitivity", Science 223:934-936 (1984). The cell yields were 18.3 $\pm$ 4.1 x 10^6 per tumor. One week later, the plates were stained with crystal violet, and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 8%-12%. The results are expressed as the surviving fraction $\pm$ SE of cells from untreated groups compared with untreated controls.

The radiation dose survival curve for the untreated FSaIIC tumor biphasic indicating two tumor subpopulations. The more sensitive cells on the first part of the curve may be the oxygenated cells and the less sensitive cells on the second part of the curve may be the hypoxic cells. When UPBHS (0.3 or 0.5 ml) was administered 1 hour before radiation treatment, a dose modifying factor of 1.5-1.7 was obtained compared with radiation and carbogen breathing.

Example III

The Eppendorf pO_2 histograph was used in the following experiment, this instrument allowed us to measure oxygen tension in tissues efficiently and

reliably. We found the rat to be the preferred animal for these studies because it is easier to maintain their body temperature and respiration rate under anesthetic and because larger solid tumor masses can be grown in them.

For our initial studies with Hemopure we used the rat 13762TB mammary carcinoma and the rat 9L gliosarocoma, both implanted subcutaneously in the hind leg of Fisher 344 female rats (200-250 grams). The pO_2 measurements made in the 13672TB mammary carcinoma are depicted in Figure 1. Under normal air breathing conditions approximately 55% of the measured points (n=1640) are at values of <5 mm Hg and the average pO_2 in the tumor is 9.3 mm Hg. Upon administration of 8 ml/kg or 12 ml/kg iv of Hemopure, the average pO_2 in the tumors increases to about 22 mm Hg (n=500 and 180, respectively). Breathing carbogen (95% O_2, 5% CO_2) is effective in increasing the oxygenation of this tumor; however, the combination of Hemopure and carbogen breathing is most effective. When the dose of Hemopure was 8 ml/kg, the percentage of the tumor at a pO_2 <5 mmHg was reduced to about 16% and the average pO_2 was increased to about 37 mmHg. With the higher does of Hemopure of 12 ml/kg, the percentage of the tumor at pO_2 <5 mm Hg was about 3% and the average tumor pO_2 was about 50 mmHg. Figure 2 graphically demonstrates that Hemopure alters the oxygenation profile of the tumor primarily by increasing the oxygen tension in the more hypoxic 50 percentile of the tumor.

Our results thus far in the rat 9L gliosarocma are even more hopeful. In the 9L brain tumor model about 46% of the tumor has a po_2 of <5 mmHg and the average pO_2 is ~7.4 mmHg under normal air breathing conditions (n=1862). (Figure 3). When Hemopure (12 ml/kg) was administered to the animals iv and normal air breathing maintained only about 13% of the tumor had a pO_2 of <5 mmHg and the average tumor pO_2 was about 18 mmHg. Carbogen is also effective in increasing the pO_2 of the 9L gliosarcoma. Under carbogen breathing conditions of 28% of the tumor had a pO_2 of <5 mmHg and the average tumor pO_2 was about 42 mmHg (n=1870). Administration of Hemopure (12 ml/kg) along with carbogen breathing further increased the oxygenation of the 9L tumor such that only about 1.5% of the tumor had a pO_2 <5 mmHg and the average tumor pO_2 was about 69 mmHg. Figure 4 graphically demonstrates that the administration of Hemopure improves the oxygenation of the more hypoxic 50 percentile of the 9L tumor whether the animals are breathing air or carbogen.

As shown on Table 2, the Hemopure preparation was more effective at enhancing the growth delay produced by the various chemotherapeutic agents. A dose of 12 ml/kg (0.3 ml/dose) appeared to be about optimal for use with the anticancer drugs.

TABLE 2. Growth delay of the FSalIC fibrosarcoma produced by various chemotherapeutic agents alone or in combination with Hemopure I or Hemopure II.

	TUMOR GROWTH DELAY, DAYS				
Treatment Group	Treatment Alone	+Hemo I (0.3 ml)	+Hemo II (0.3 ml)	+Hemo II (0.5 ml)	+Hemo I (1.0 ml)
Cyclophosphamide (3 x 150 mg/kg)	7.8	9.7	22.9	26.2	12.3
melphalan (10 mg/kg)	3.1	6.9	11.5	10.7	8.8
cisplatin (10 mg/kg)	4.4	5.9	6.9	7.1	5.7
carboplatin (3 x 50 mg/kg)	4.3	7.4	9.4	8.6	7.4
etoposide (3 x 15 mg/kg)	2.8	3.8	9.5	8.8	3.42
5-fluorouracil (5 x 40 mg/lg)	7.6			10.9	

CLAIMS

1. A method for treating a tumor in a host with ionizing radiation, comprising:
   a) administering to said host an ultrapurified polymerized hemoglobin solution in an amount sufficient to significantly increase the antitumor effect of said ionizing radiation; and,
   b) administering to said tumor an effective amount of said ionizing radiation.

2. A method of Claim 1 wherein said ionizing radiation comprises X-rays, gamma rays, electrons or high LET radiation.

3. A method of Claim 1 wherein said hemoglobin comprises a hemoglobin which is dependent upon chloride ion concentration for oxygen transport.

4. A method of Claim 1 wherein said hemoglobin is bovine hemoglobin.

5. A method of Claim 1 wherein said ionizing radiation comprises X-rays, gamma rays or electrons.

6. A method of Claim 1 wherein said host is a mammal.

7. A method of Claim 6 wherein said mammalian host is a human being.

8. In a method of treating a tumor in a mammalian host with ionizing radiation:
The improvement of administering to said mammalian host, prior to treatment with said ionizing radiation, an ultrapurified bovine hemoglobin solution in an amount sufficient to significantly increase the antitumor effect of said ionizing radiation.

9. the improvement of Claim 8 wherein said mammalian host comprises a human being.

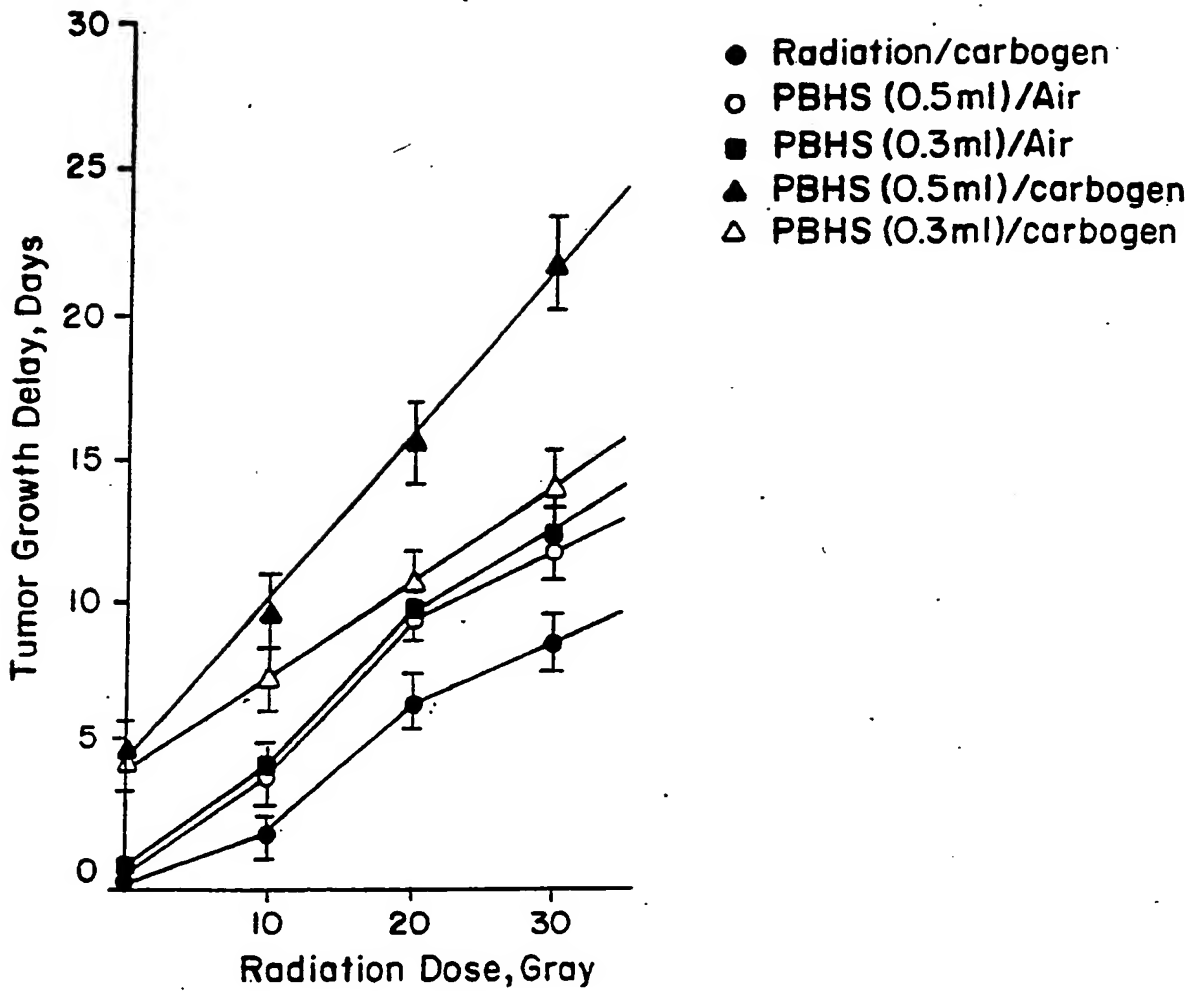
Tumor Growth Delay, Days
30
25
20
15
10
5
0
10
20
30
Radiation Dose, Gray
Radiation/carbogen
PBHS (0.5ml)/Air
PBHS (0.3ml)/Air
PBHS (0.5ml)/carbogen
PBHS (0.3ml)/carbogen

FIG. 1A

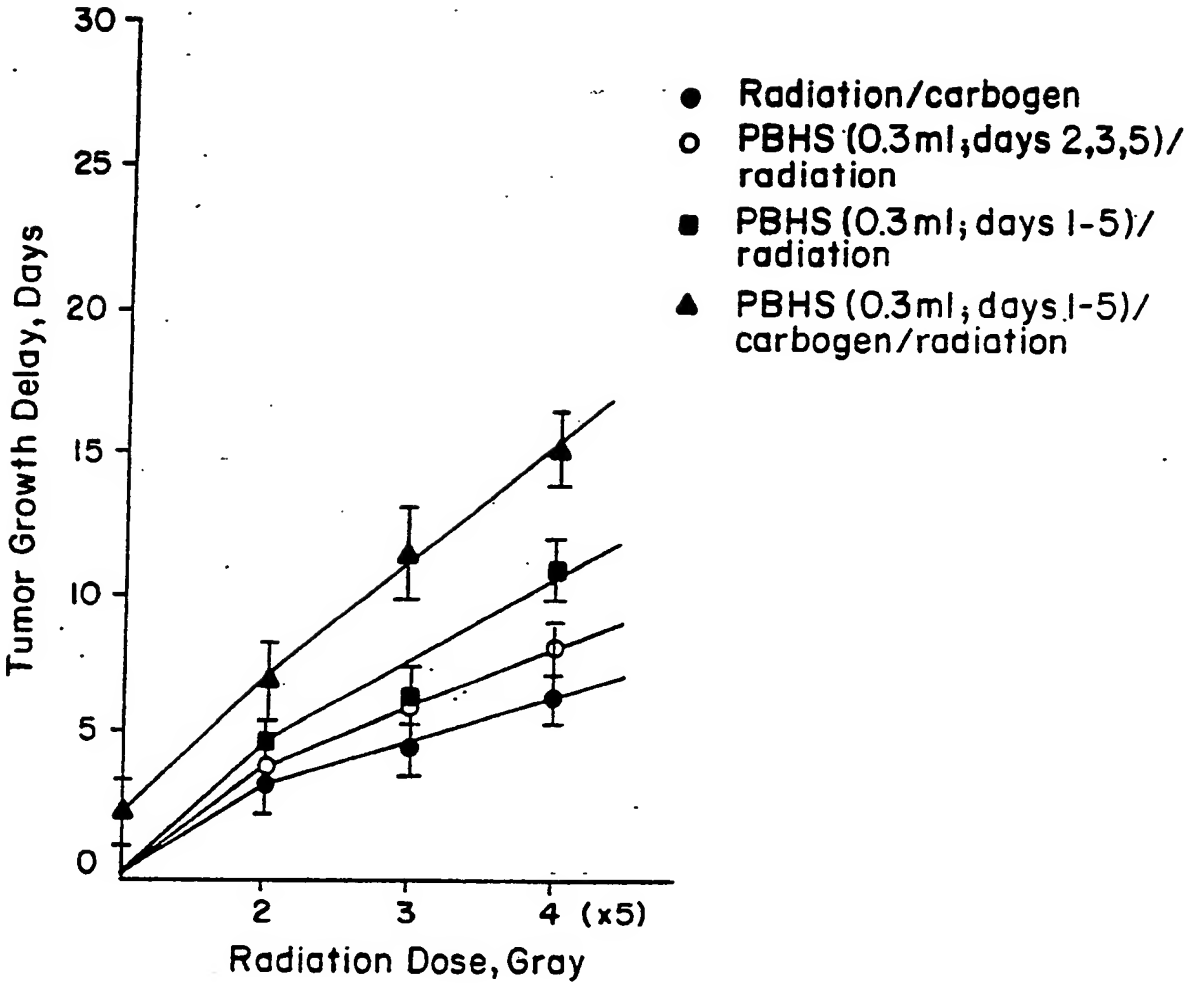
30
25
20
15
10
5
0
Tumor Growth Delay, Days
2
3
4 (x5)
Radiation Dose, Gray
Radiation/carbogen
PBHS (0.3ml;days 2,3,5)/ radiation
PBHS (0.3ml; days 1-5)/ radiation
PBHS (0.3ml; days 1-5)/ carbogen/radiation

FIG. 1B

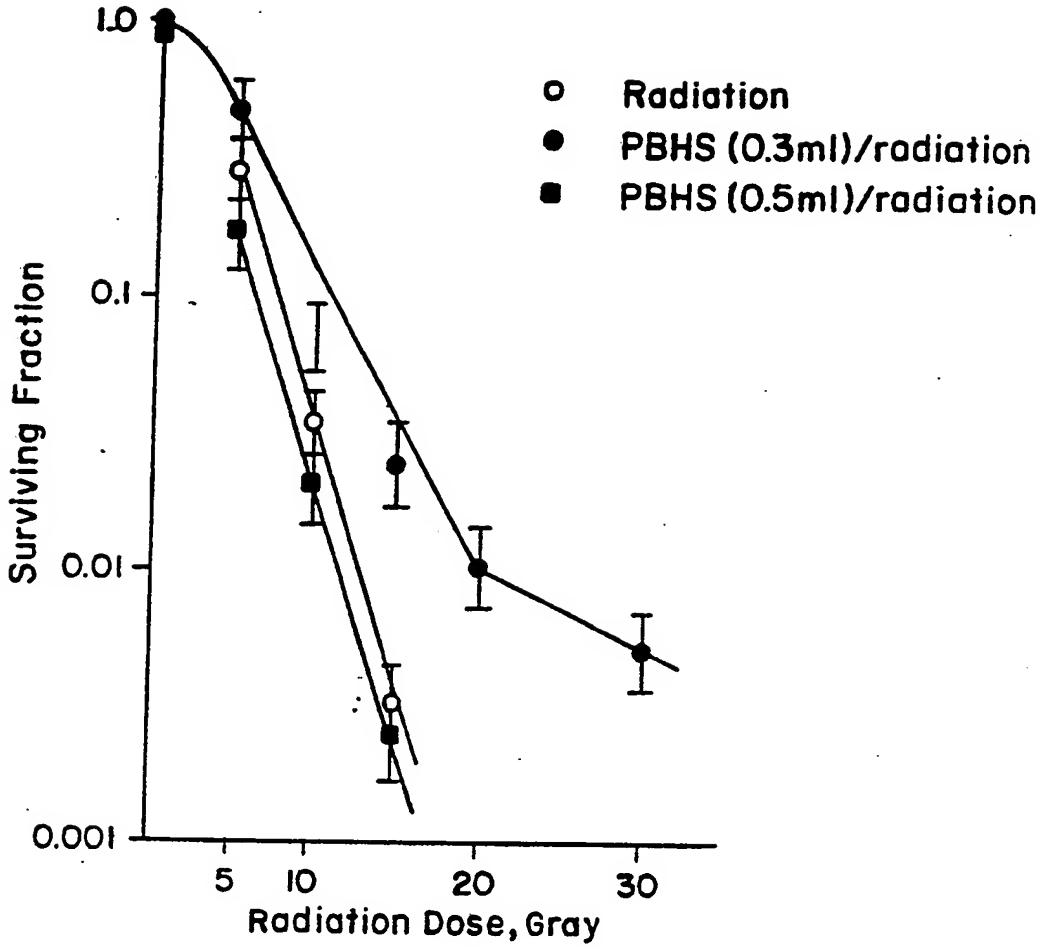
1.0
0.1
0.01
0.001
Surviving Fraction
5
10
20
30
Radiation Dose, Gray
Radiation
PBHS (0.3ml)/radiation
PBHS (0.5ml)/radiation

FIG. 2

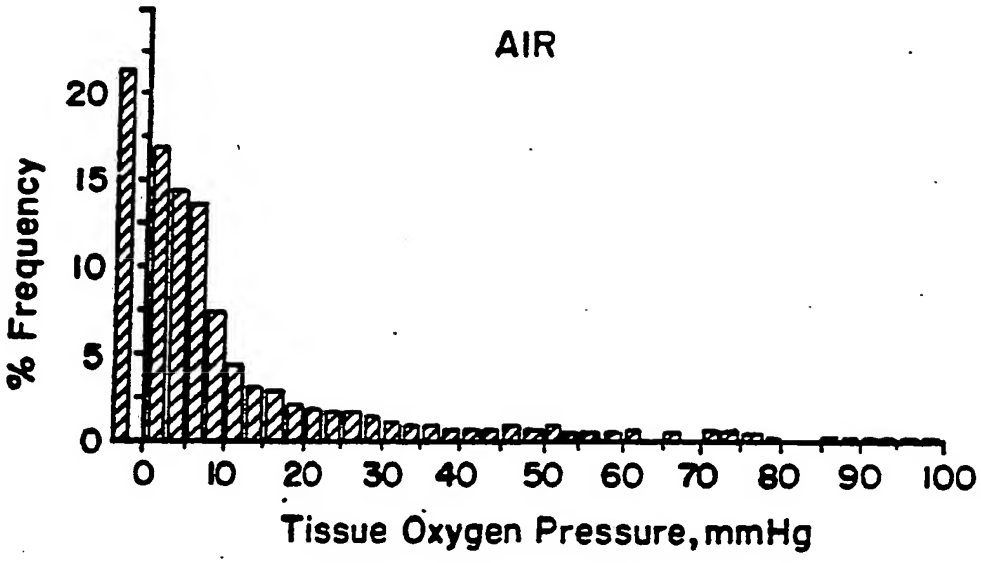
AIR
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3A

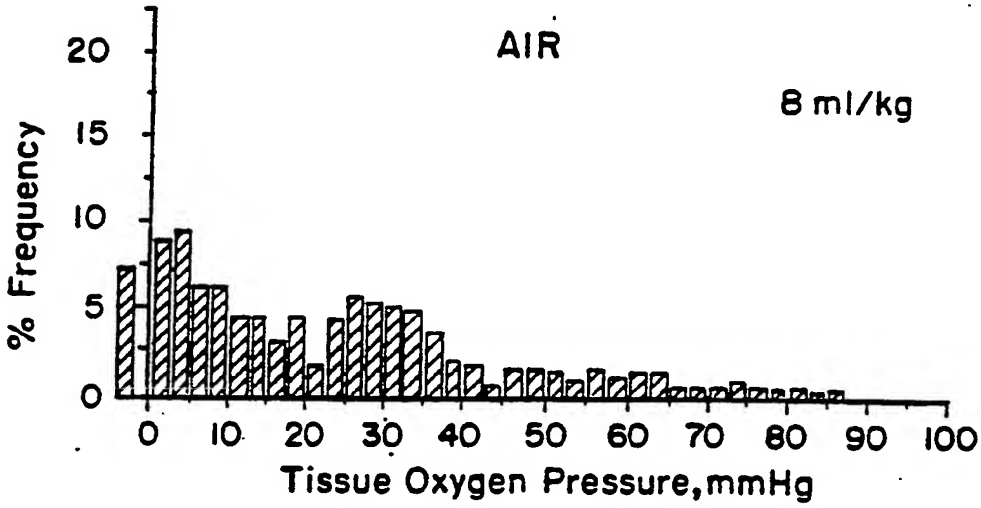
AIR
8 ml/kg
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3B

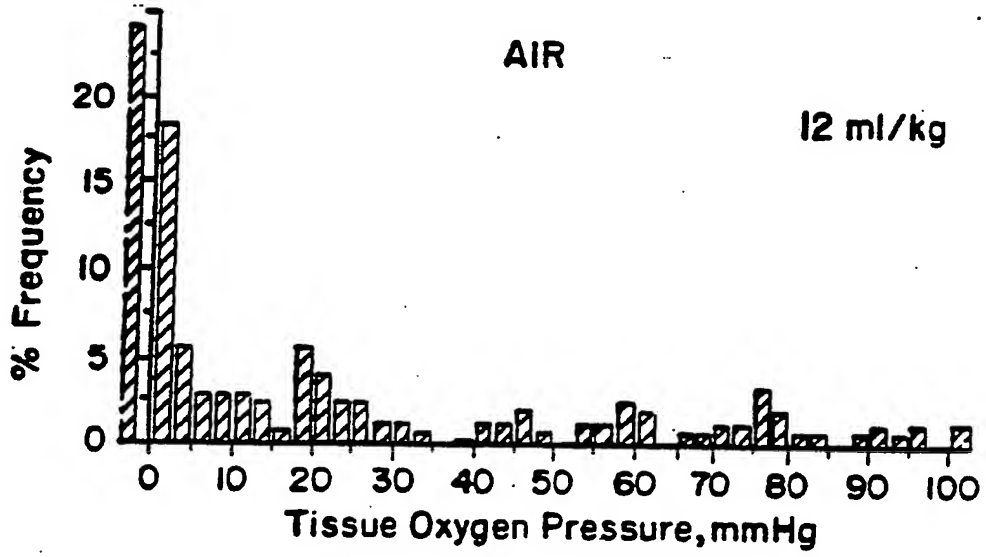
AIR
12 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3C

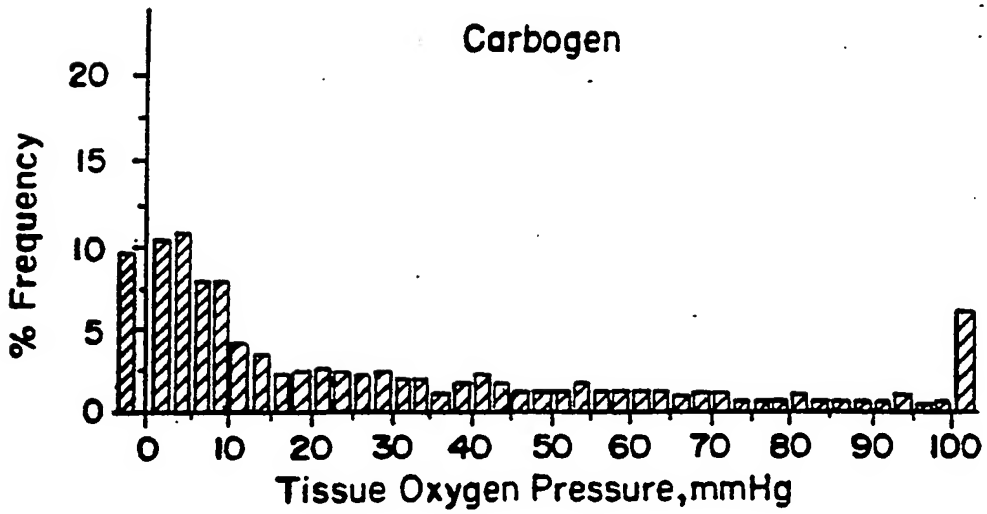
Carbogen
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3D

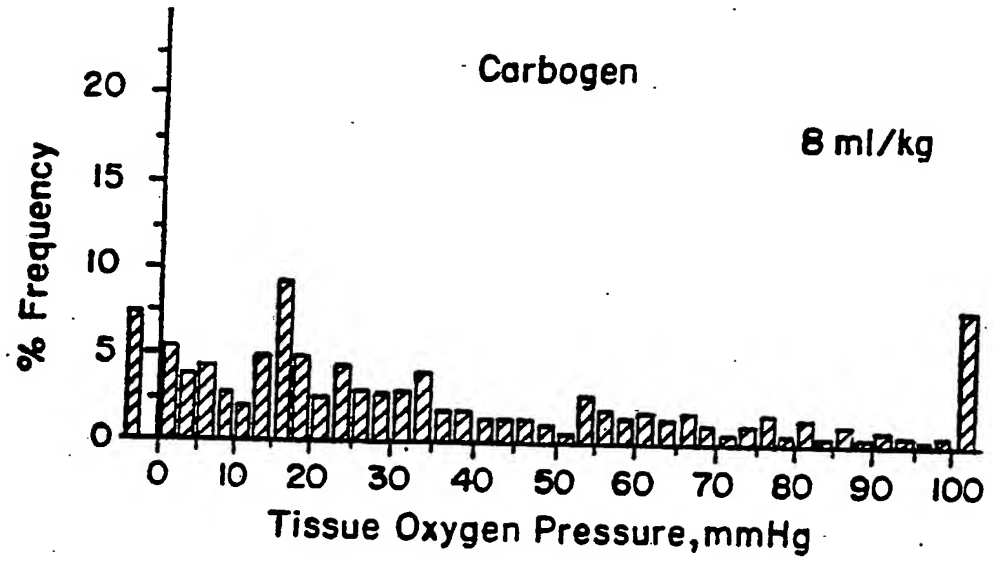
Carbogen
8 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3E

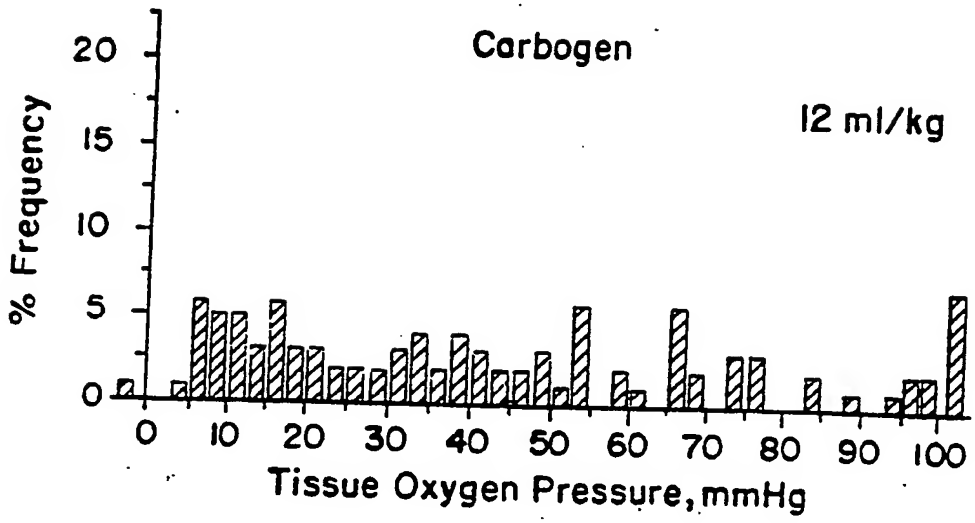
Carbogen
12 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3F

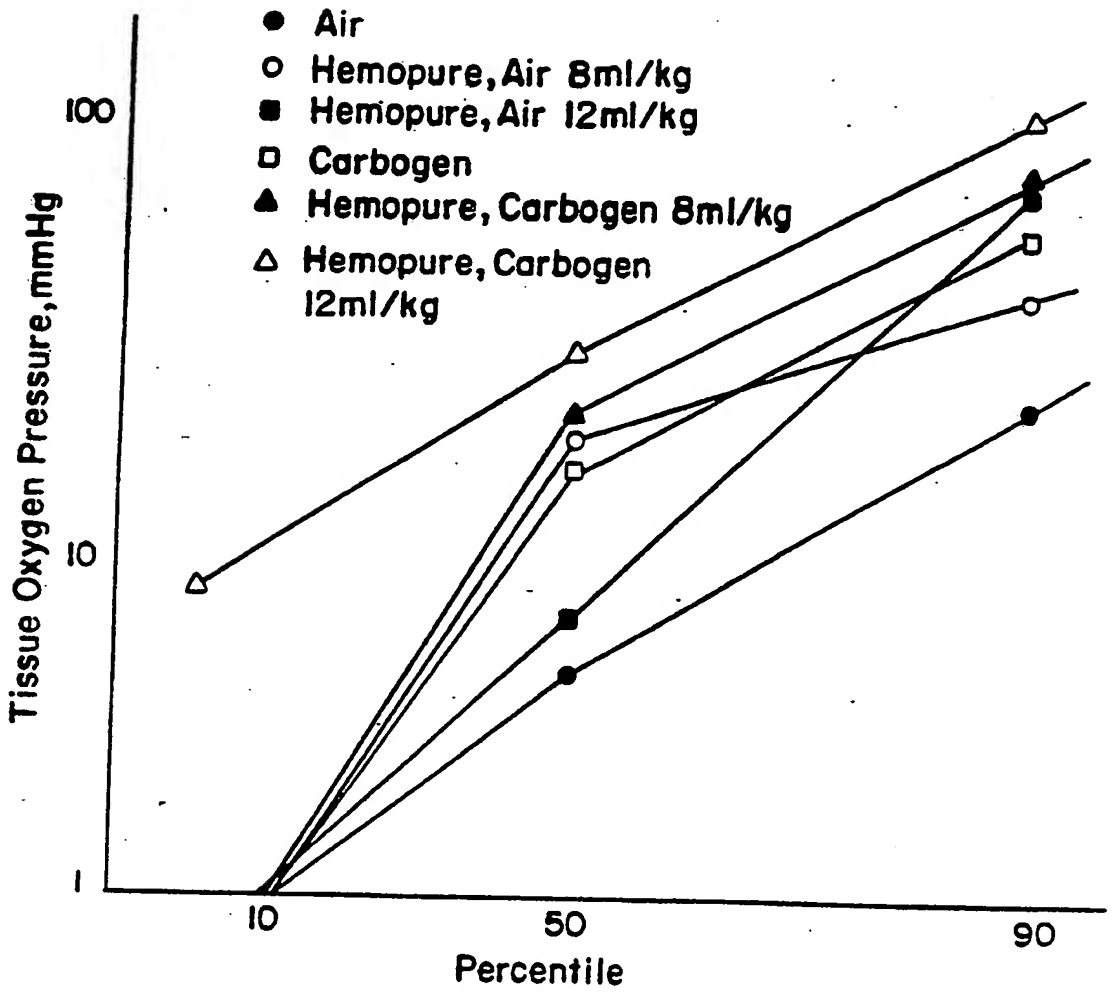
Air
Hemopure, Air 8ml/kg
Hemopure, Air 12ml/kg
Carbogen
Hemopure, Carbogen 8ml/kg
Hemopure, Carbogen 12ml/kg
Tissue Oxygen Pressure, mmHg
100
10
1
10
50
90
Percentile

FIG. 4

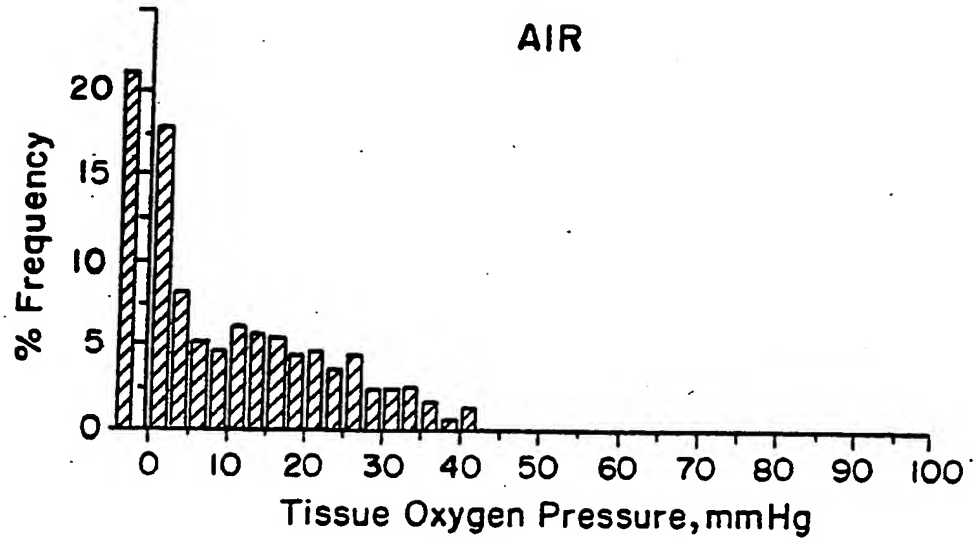
AIR
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5A

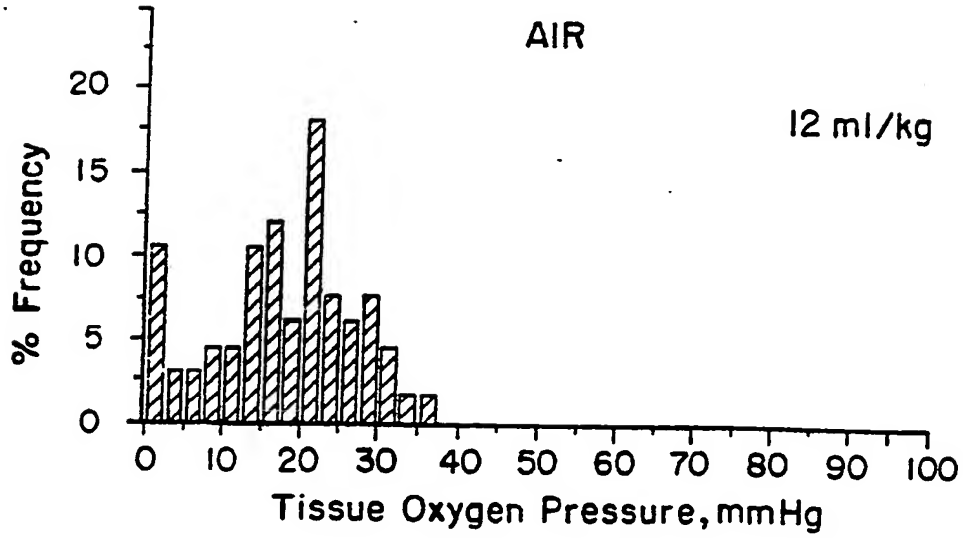
AIR
12 ml/kg
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5B

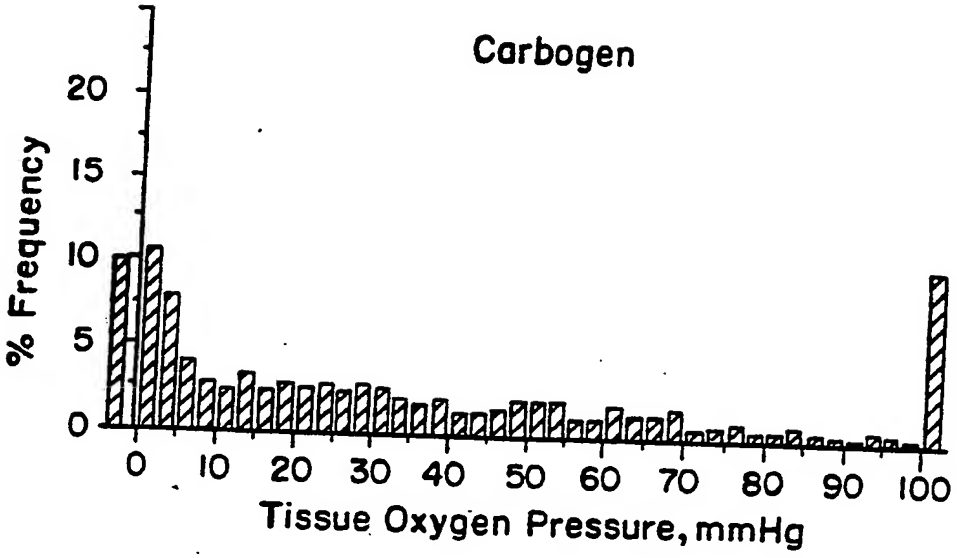
Carbogen
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5C

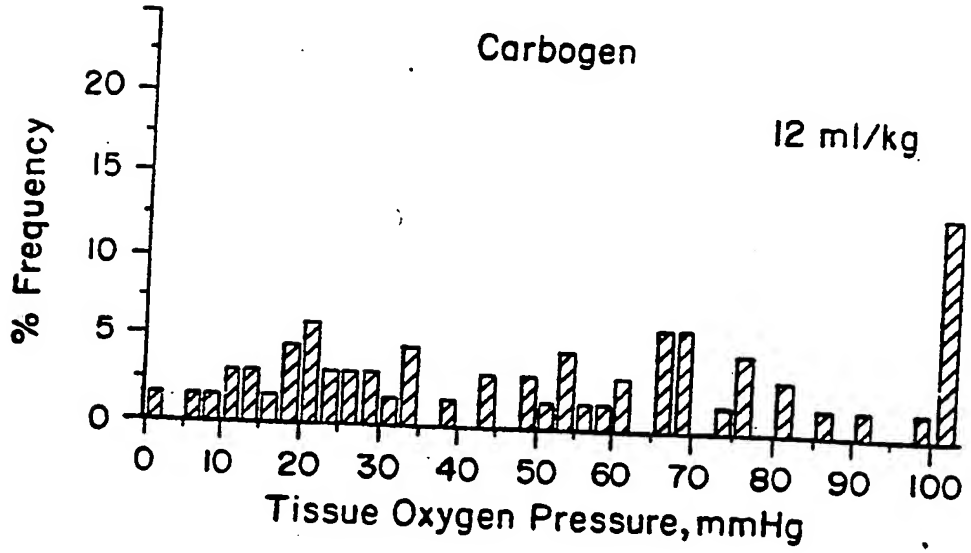
Carbogen
12 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5D

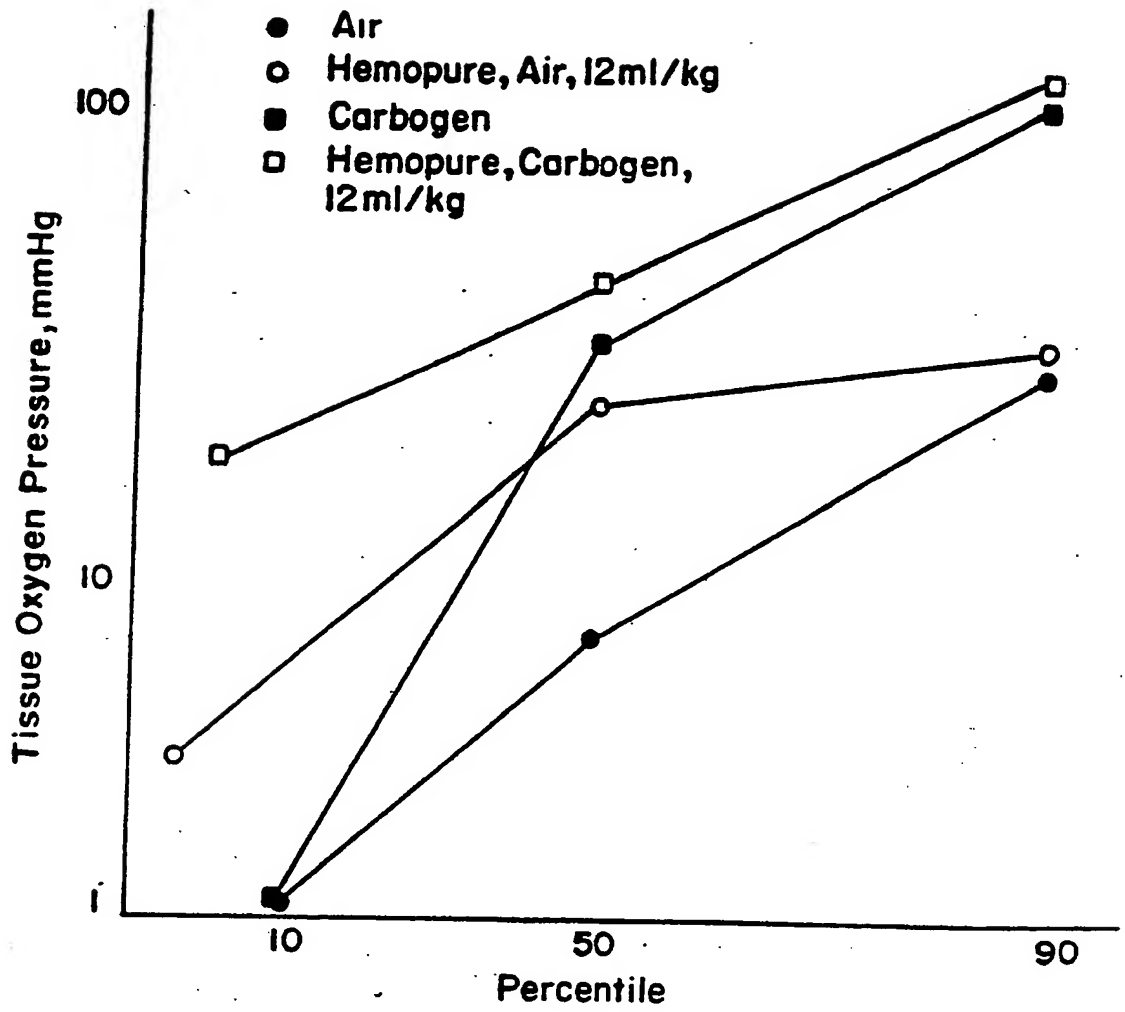
Air
Hemopure, Air, 12ml/kg
Carbogen
Hemopure, Carbogen, 12ml/kg
100
10
1
Tissue Oxygen Pressure, mmHg
10
50
90
Percentile

FIG. 6

NTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04067

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/14; A61K43/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS vol. 19, no. SUP1, 1990, NEW YORK page 137; TEICHER B.A. ET AL: 'Effect of oxygen level on the enhancement of tumor response to radiation by perfluorochemical emulsions(pfces) or hemoglobin' see abstract 27 --- -/—	1-9

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 SEPTEMBER 1992	29. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	FERNANDEZ Y BRA F.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,O	INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS vol. 21, no. 4, September 1991, NEW YORK pages 969 - 974; TEICHER B.A. ET AL: 'Effect of oxygen level on the enhancement of tumor response to radiation by perfluorochemical emulsions or a bovine hemoglobin preparation' Presented in part at the american society for therapeutic radiation and oncology annual meeting, Miami beach, FL, october 1990 see the whole document	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04067

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1 - 9 are directed to a method of treatment of the human or animal body, the search has been carried out and based on the alleged effects of the composition

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.